

Enhanced oral availability/pheromonotropic activity of peptidase-resistant topical amphiphilic analogs of pyrokinin/PBAN insect neuropeptides

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Abstract

The peptide bond between active core residues Pro and Arg is the primary site of susceptibility for the pyrokinin/PBAN neuropeptides to insect tissue-bound peptidases, and incorporation of modified Pro residues can enhance resistance to peptidase hydrolysis. An Hyp-containing amphiphilic analog (Hex-FT[Hyp]RLa) is shown to operate as a topically active tissue-bound peptidase-resistant analog of the pyrokinin/PBAN class of insect neuropeptides in adult *Heliothis virescens* moths. An Oic amphiphilic analog (Hex-FT[Oic]RLa) is ineffective topically, but proves to be a superior tissue-bound, peptidase-resistant pyrokinin/PBAN analog for oral administration; outperforming both the Hyp analog and the orally inactive natural hormone PBAN in the moths. The Oic analog is effective in penetrating an isolated, ligated foregut preparation, but less successful in transmigrating an isolated midgut preparation; whereas the opposite behavior is observed for the Hyp analog. The success of the Oic analog via oral administration may be related to its ability to effectively penetrate the foregut, thereby bypassing the hostile environment of the midgut region.

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1. Introduction

Insect neuropeptides are important regulators of insect physiology and behavior [5,16] critical for survival and efficient propagation. As potent and specific as these molecular messengers are, neuropeptides in and of themselves are not suitable as pest insect control agents and/or tools for insect neuroendocrinologists. Direct use of neuropeptides for insect control is impractical because the insect cuticle contains an apolar lipid matrix that inhibits penetration of polar compounds like peptides and because the insect gut, hemolymph and membranes of a number of tissues contain peptidases that rapidly degrade the peptides [7,9]. The development of agonists and antagonists with enhanced peptidase-resistance and cuticle/gut penetrability can overcome these limitations and represents a key step in the development of control techniques employing analogs of insect neuropeptides capable of disrupting critical life processes.

We have addressed these problems separately through the development of pseudopeptide analogs of one class of

insect neuropeptides (pyrokinin/PBAN class) with amphiphilic character, which confers an ability to penetrate the hydrophobic insect cuticle and simultaneously maintains the water solubility necessary for them to re-emerge in the insect circulatory system and reach their target receptor sites [2,12–14,17,19]. Peptidase-resistant analogs of two other classes of insect neuropeptides have been shown to demonstrate enhanced hemolymph residence time and in vivo biological activity [7,11]. However, analogs featuring both enhanced cuticle-penetration and peptidase-resistance have not yet been developed and will be addressed in this paper.

We have been developing analogs of neuropeptides that penetrate the insect cuticle for some time using the pyrokinin/PBAN class of insect neuropeptides as models for analog development [2,12–14,17,19]. This class of neuropeptides regulates numerous physiological events in insects including hindgut and oviduct contraction in cockroaches and flies, induction of egg diapause, reddish coloration and melanization in moths, pupariation acceleration in flies, and sex pheromone biosynthesis in moths and some flies [9]. These neuropeptides range in length from 8–34 amino acids and share a common C-terminal

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pentapeptide Phe-Xxx-Pro-Arg-Leu-NH₂ (Xxx = Gly, Ser, Thr, or Val). This common sequence forms a type I β -turn required for receptor recognition and is the critical portion of these peptides required for bioactivity in virtually all physiological assays [8,10]. Studies have demonstrated that the most potent of these pentapeptides in pheromonotropic assays is Phe-Thr-Pro-Arg-Leu-NH₂ [1]. Analog design has involved the attachment of lipid moieties to the amino terminus of the aforementioned pentapeptide, which imparts amphiphilic character to the analogs, demonstrating both water solubility and cuticle penetrability [2,11–14,17,19].

In this study, we investigate the regions within the C-terminal pyrokinin pentapeptide core most susceptible to hydrolysis by tissue-bound peptidases and develop amphiphilic analogs incorporating residues that protect the susceptible regions. The pheromonotropic potency and ability to penetrate the cuticle of the tobacco budworm moth *Heliothis virescens* is then ascertained for the analogs. In addition, the oral availability of these peptidase-resistant, amphiphilic pyrokinin/PBAN analogs is probed by looking at direct passage of the analogs through isolated insect mid- and foregut, and finally measurement of pheromone production following oral ingestion in adult *H. virescens* moths.

2. Materials and methods

2.1. Pseudopeptide synthesis

The pyrokinin amphiphilic pseudopeptides were synthesized via condensation of the fatty acid component hexanoic acid (Aldrich Chemical Co., Milwaukee, WI) with the peptide-resin complexes Phe-Thr(OtBu)-Hyp(OtBu)-Arg(Pmc)-Leu-Rink Amide (Hyp, hydroxyproline; tBu, tertiary butyl; Pmc, pentamethylchroman-6-sulfonyl) and Phe-Thr(OtBu)-Oic-Arg(Pmc)-Leu-Rink Amide (Oic, octahydroindole-2-carboxyl). Each peptide-resin was synthesized via Fmoc solid phase technology on an ABI 433A peptide synthesizer (Applied Biosystems, Foster City, CA) according to previously described procedures [11,14] using one equivalent of a 1,3-diisopropylcarbodiimide/1-hydroxy-7-azabenzotriazole mixture in dimethylsulfoxide for 4 h according to previously described procedures [14]. Protected amino acid starting materials were purchased from Advanced Chemtech (Louisville, KY) or, in the case of Fmoc-Hyp(OtBu)-OH and Fmoc-Oic-OH, from Bachem Americas (King of Prussia, PA). The pseudopeptide crude products were cleaved from the resin complex and deprotected by stirring with a mixture of trifluoroacetic acid (95%), anisole (5%), thioanisole (4%), and 1,2-ethanedithiol (1%) for 1 h at RT. The resin was filtered and volatile reagents were removed in vacuo on a Savant Speed Vac concentrator at 40 °C.

The products were purified on a Waters C18 Sep Pak cartridge and a Delta Pak C18 reverse-phase column at

ambient temperature on a Waters Model 510 HPLC controlled with a Millennium 2000 chromatography manager system (Waters, Milford, MA) with detection at 214 nm. Solvent A = 0.1% aqueous trifluoroacetic acid (TFA); solvent B = 80% aqueous acetonitrile containing 0.1% TFA. Conditions: initial solvent consisting of 20% B was followed by a linear program to 100% B over 40 min; flow rate: 2 ml/min. Retention times on C18 column: 19.25 min; Hex-Phe-Thr-Hyp-Arg-Leu-NH₂ (901), 15.75 min; and Hex-Phe-Thr-Oic-Arg-Leu-NH₂ (904), 18.00 min. Two of the analogs required further purification on a Waters Protein Pak 125 column. Solvent A = 95% aqueous acetonitrile containing 0.01% TFA; solvent B = 50% aqueous acetonitrile containing 0.01% TFA. Conditions: 100% A isocratic for 4 min followed by linear program to 100% B over 8 min. Retention times on Protein Pak 125 column: Hex-Phe-Thr-Oic-Arg-Leu-NH₂ (904), 7.50 min. The pure peptides were analyzed and quantified via amino acid analysis. Each peptide sample was purged with N₂ and the peptide hydrolyzed with vapor phase HCl for 24 h at 105 °C. Pre-column derivatization and HPLC analysis was accomplished by the standard Pico Tag method supplied by Waters (Milford, MA). The observed amino acid ratios were as expected for each analog: Hex-Phe-Thr-Hyp-Arg-Leu-NH₂ (901), F[1.0], L[1.0], R[1.0], T[1.0]; and Hex-Phe-Thr-Oic-Arg-Leu-NH₂ (904), F[1.0], L[1.0], R[1.0], T[0.9]. Mass spectra were obtained on a Kratos Kompact Probe MALDI-TOF machine (Kratos Analytical, Ltd., Manchester, UK) using α -cyano-4-hydroxycinnamic acid as a matrix, recording the presence of the following molecular ions: Hex-Phe-Thr-Hyp-Arg-Leu-NH₂ (901), 747.1 [calculated MH⁺ = 747.53]; and Hex-Phe-Thr-Oic-Arg-Leu-NH₂ (904), 784.66 [calculated MH⁺ = 784.48].

2.2. Tissue-bound peptidase susceptibility assays

Malpighian tubules and/or hindguts were dissected from adult males of the corn earworm *Heliothis zea* (*Heliothis* premix artificial diet purchased from Stonefly Industries, Inc., Bryan, TX) and were incubated with 5 nmol peptide analog in 500 μ l *Manduca* saline [3] for 5 min up to 4 h. At the end of each incubation period, the tubules and/or hindguts were removed and 500 μ l 15% aqueous TFA was added to the tube. The tubes were then vortexed and centrifuged, and the resulting supernatant was run on reversed phase HPLC Deltabond C18 ODS 250 \times 4.6 mm, 5 μ M, 300 Å; A solvent: 0.1% TFA; B solvent: 80% acetonitrile in 0.1% TFA, 0–100%/80 min; 1 ml/min.). The experiment was repeated a minimum of four times. Fractions were collected, dried, and analyzed on a Kratos Kompact Probe MALDI-TOF mass spectrometer (Kratos Analytical, Manchester, UK) to identify degradation products. Masses of naturally cleaved peptide fragments were compared with calculated masses of putative degradation products. The progress of the peptidase degradation was followed by monitoring the disappearance of the substrate peak.

2.3. *Heliothis virescens*

Heliothis virescens moths were obtained as pupae from North Carolina State University. Pupae were sexed and males discarded. Females were held in 30 cm³ plastic cages equipped with mesh sleeves to allow for removal of adults. Newly eclosed adults were placed in new cages daily and held in environmental chambers at 25 °C, 65% relative humidity, a 14 h:10 h light:dark photoperiod, and provided with a 5% sucrose solution on cotton wicks. All bioassays were conducted using 3-day old females during the photophase, when endogenous levels of pheromone are low or undetectable [1]. Adult females of the American cockroach were obtained from a colony maintained at CMAVE and were held in 11 glass jars having screen tops. Cockroaches were provided with dry dog food and water and maintained under the same conditions as described for moths.

2.4. Pheromonotropic assay (injection)

Pheromonotropic assays were conducted using *Heliothis virescens* moths. We assessed the effect of the pseudopeptides in inducing sex pheromone biosynthesis by injecting different amounts of the pseudopeptides dissolved in 10 µl of saline into females and comparing the amounts of pheromone produced to amounts produced when females were injected with 5 pmol/10 µl water of PBAN or just saline [1]. After incubation, the sex pheromone glands were excised from the insects and extracted in 2 µl of hexane containing 1 ng/µl of heptadecane and nonadecane as internal standards. The amount of pheromone in individual extracts was determined by quantifying the amount of (Z)-11-hexadecenal (Z11-16:AL) using capillary gas chromatography as described elsewhere [1]. The amount of pheromone in extracts obtained from insects injected with the pseudopeptide or with saline only were converted to a percentage of the mean amount present in extracts obtained from females injected with 5 pmol of PBAN for that day.

2.5. Pheromonotropic assay (topical)

We conducted assays to determine the effects of topical application of the pseudopeptides in inducing pheromonotropic responses. For these assays, we removed the scales on the surface of the abdomen by gently dabbing the ventral surface of the abdomen on cellulose adhesive tape. Moths were held immobile, ventral side up, by clamping the wings behind the back using smooth jawed alligator clips held in modeling clay. A 1-µl drop of H₂O containing between 1 and 1000 pmol of the analog was applied to the de-scaled portion of the abdomen [2], and the insects were incubated for various times. After incubation, the pheromone glands were excised, extracted, and the extracts analyzed as above. Control treatments for this experiment consisted of abdomens treated with only water and females injected with

5 pmol of PBAN and incubated for 1 h prior to excision and extraction of the pheromone glands.

2.6. Isolated cuticle penetration assay

Cuticle penetration assays were conducted using tissue obtained from the abdomen of female moths. Pieces of cuticle, ca. 0.4 cm², were prepared as described elsewhere [17] and floated cell-side down in wells of ELISA plates (Corning, 96-well Easy Wash) that had been previously blocked by filling with 1% gelatin in 10 mM sodium phosphate buffer containing 150 mM NaCl (pH 7.25) [17]. Either 901 or 904 (0.5 nmol) were applied to the center of the cuticle pieces in a 0.25 µl drop of H₂O using a 10-µl syringe and observed for 5 min to insure that the drops did not slide off. Lids were applied to the plates and the plates were placed on an orbital shaker operated at 80 rpm. One hour after application of the analogs, we carefully transferred the cuticle to new wells containing 300 µl of H₂O and incubation was continued. Cuticle was subsequently transferred to new wells at 2, 4, 6, 8, 21, and 24 h after application of the analogs. After incubation, 100 pmol of internal standard (6Pha-FTPLA; 6Pha, 6-phenylhexanyl) were added and the contents of the wells were prepared for reversed phase liquid chromatographic analysis (see [17]). Samples were analyzed as described below.

2.7. Isolated fore- and midgut penetration assays

We studied penetration of the pseudopeptide analogs through the fore- and midgut of the American cockroach because the guts of adult moths are not amenable to study. Cockroaches were anesthetized by placing them in tepid water for 10 min. Then the entire alimentary canal was dissected out from the animal and carefully cleared of extraneous tissues under saline. The alimentary canals were transferred to fresh saline and the gut contents carefully flushed out using saline delivered using a 5-ml syringe fitted with a 22-gauge nylon cannula. The fore- and midguts were removed and placed in separate saline filled dishes. Tight ligatures were attached to the posterior end of either gut sections using Teflon thread and a Teflon tube (22 gauge) affixed to the needle of a 25-µl gas tight syringe was inserted through the anterior opening of the gut prior to tightly affixing another ligature at the anterior end around the gut and Teflon tube. The anterior end of the preparation was elevated and 5 µl of saline containing 500 pmol/µl of either of the pseudopeptides or PBAN were injected into the gut lumen. The Teflon tube was removed and the ligature tightened. The preparation, resembling a sausage bound at both ends, was placed in a vial containing saline on a rotary mixer at 80 rpm for 2 min and then dipped in another saline bath for 1 min to remove pseudopeptide or peptide on the gut surface. The tissue was then placed in 200 µl of saline on the rotary mixer (80 rpm) and incubated for 4 h. After 4 h, the tissue was transferred to fresh saline and incubated for

an additional 20 h. Immediately after removal of tissue from incubation media, we added 200 μ l of acetonitrile to the media and mixed well followed by addition of 400 μ l of water containing 1 nmol of 6Pha-FTPRLa (see [17]) as an internal standard. Samples were stored at -20° until analysis.

Samples were analyzed by reversed phase liquid chromatographic analysis (RPLC) using a LDC Biochrome quaternary gradient pump and LDC Spectro Monitor 3200 UV detector set at 210 nm and interfaced to a Nelson Analytical 3000 data acquisition and analysis system. A Macrosphere 300 C18 reversed phase column (250 mm \times 2.1 mm i.d., 5 μ m, Alltech) was used for all separations. Solvents used for all separations were H₂O and MeCN each containing 0.1% TFA as the ion pair reagent. Samples were injected onto the column using a Rheodyne 7125 injector (250 μ l loop) in 25% MeCN. The column was eluted after a 5 min equilibration period using a linear gradient from 25–75% MeCN over 90 min at a flow rate of 0.25 ml/min. Quantitation was accomplished by comparing the areas of the pseudopeptides or PBAN to that of the internal standard, 6Pha-FTPRLa. However, analysis of equimolar amounts of pseudopeptides, PBAN and the internal standard, 6Pha-FTPRLa, indicated that the compounds had different detector responses when analyzed with the UV detector set at 210 nm. Therefore, all values were corrected to reflect the differential detector response for the compounds.

Data were analyzed using a one-way ANOVA and Fishers Least Significant Difference test using NCSS7 Statistical software and by regression analysis using GraphPad Prism[®] software.

2.8. Pheromonotropic assay (oral)

Initially we conducted experiments to determine if PBAN or FTPRLamide would induce pheromonotropic responses when 5, 25, or 50 pmol were fed to female moths. These experiments were conducted as described by Raina et al. [15] except that we sampled pheromone glands both 1 and 2 h after the moths finished drinking. Controls for these experiments included females that were given only sugar water to drink and females that were injected with 5 pmol of PBAN and incubated for 1 h prior to excision and extraction of the pheromone gland. These studies failed to induce a pheromonotropic response. Therefore we developed an alternate method to deliver the test substances orally. In these studies we starved 2-day old females for 18 h. During the middle of the photophase of day 3, females were held immobile, dorsal side up, by clamping the wings behind the back with smooth jawed alligator clips and the clips were positioned in modeling clay so that the moths were at a 45° angle. The tops of 500 μ l polypropylene microcentrifuge tube were used to hold 30 μ l of a 10% sucrose solution containing 50 pmol/ μ l of one or the other of the peptides or pseudopeptides. The tip of the centrifuge tube was positioned in modeling clay at an angle of 45° and females were moved close to the tube so that the front feet touched the

cap of the tube. Females were induced to feed on the liquid by carefully placing the tip of the proboscis into the liquid using watchmakers forceps. After 15 min, the centrifuge tubes were removed and the alligator clips removed from the females who were placed in cages for various periods of time. After incubation, the pheromone glands were excised, extracted and analyzed as above. Only females who consumed all 30 μ l of solution were sampled. Controls included equal numbers of females fed only 30 μ l of sugar water and females who were injected with 5 pmol of PBAN and incubated for 1 h prior to extraction of the pheromone gland.

3. Results

3.1. Tissue-bound peptidase susceptibility assays

Fig. 1 illustrates the two modified Pro residues used in pseudopeptide analog sequences used in this study in

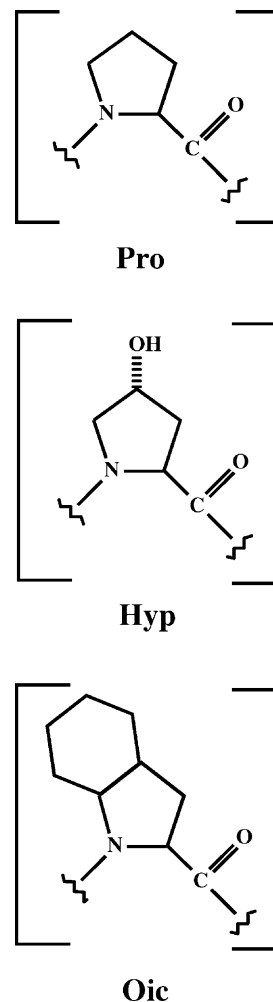


Fig. 1. Structures of modified Pro residues incorporated into pyrokinin/PBAN pseudopeptide analogs to enhance resistance to insect tissue-bound peptidases. Pro (top), hydroxyproline (Hyp) (middle), and octahydroindole-2-carboxyl (Oic) (bottom).

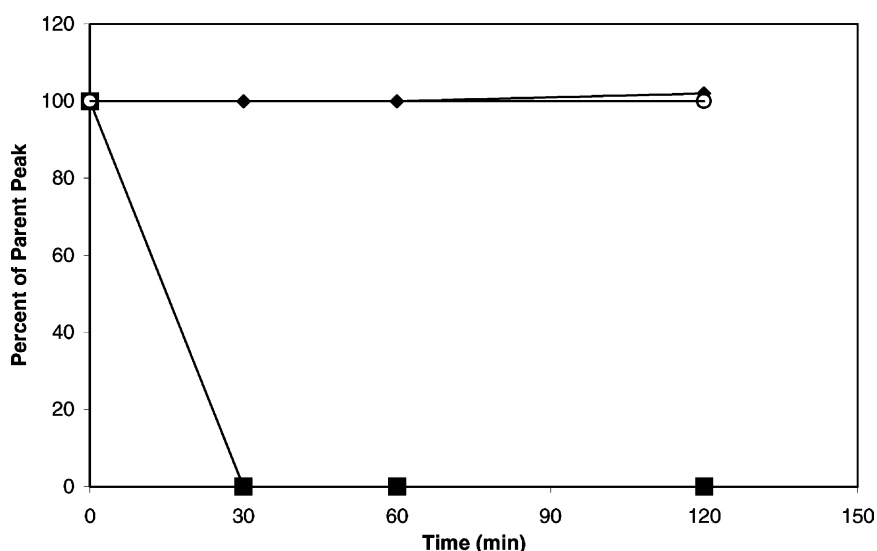


Fig. 2. Stability of the natural pyrokinin/PBAN analog LPK (□), and the peptidase-resistant pyrokinin/PBAN analogs Hex-Phe-Thr-Hyp-Trp-Gly-NH₂ (901) (○) and Hex-Phe-Thr-Oic-Trp-Gly-NH₂ (904) (◆) to hydrolysis by peptidases bound to corn earworm (*H. zea*) Malpighian tubule tissue. Measurement of the amount of remaining peptide was made by HPLC at 30, 60, and 120 min. The data points represent the means of at least three replicates.

comparison with the C-terminal pentapeptide sequence of the pyrokinin LPK [6], containing a Pro in position 3. As can be visualized in Fig. 2, LPK is rapidly degraded by the *H. zea* Malpighian tubule tissue and completely removed in as short a time as 30 min. Analysis of the fragments by MALDI mass spectrometry indicate that the primary site of hydrolysis is the peptide bond between the Pro and Arg residues. The primary fragment observed was pQTSFTP-OH. Analogs incorporating Hyp and Oic substitutions (Fig. 1) for the Pro demonstrate enhanced resistance to degradation by the tissue-bound peptidases. Even up to a 2 h exposure, each of these analogs is stable to hydrolysis (Fig. 2).

3.2. Pheromonotropic assay (injected)

Injection of either of the two analogs induced production of pheromone in dose-dependent fashions (Table 1). However, the analog 901 was more potent than analog 904 and induced a maximal pheromonotropic response at a dose of 50 pmol (ED₅₀ calculated = 9.0 pmol). Optimal production 1 h after injection of analog 904 required injection of at least 125 pmol (ED₅₀ calculated = 113.5 pmol; Table 1). By comparison, the ED₅₀ of PBAN and its C-terminal pentapeptide (FTPRLamide) is 1.0 pmol (maximum = 2.5 pmol) and 2.3 pmol (maximum = 10 pmol), respectively. The effects of the analogs in inducing pheromone production over time were conducted by injecting the minimal dose required to stimulate maximum production of pheromone in 1 h (50 pmol of 901 and 125 pmol of 904). The results showed that the analogs had an activity period of only 3 h with maximum production occurring between 2–3 h for both (data not shown).

3.3. Pheromonotropic assay (topical)

Topical application studies indicated that pheromone production could not be stimulated by application of as much as 1000 pmol of 904. However, application of 500 pmol of 901 induced production of 43 ± 12.3 ng of pheromone in 1 h. This was significantly greater than the 6.87 ± 1.9 ng present in extracts of glands obtained from control insects treated with water and 35% of the amount from extracts obtained 1 h after injection of PBAN. To determine if increased time after application of the analogs affected activity of the analogs we applied 500 pmol and sampled glands at 2–8 h after application. As indicated in Fig. 3, only 901 induced pheromone production, and optimal production was achieved 4 h after application.

Table 1
Comparative injected pheromonotropic activity of peptidase-resistant, amphiphilic pyrokinin analogs with PBAN and the C-terminal pentapeptide

Peptide analog	ED ₅₀ (pmol)	Maximal response ^a (pmol)
Hex-Phe-Thr-[Hyp]-Arg-Leu-NH ₂ (901) ^b	9	50
Hex-Phe-Thr-[Oic]-Arg-Leu-NH ₂ (904) ^b	114	125
Phe-Thr-[Pro]-Arg-Leu-NH ₂ (pyrokinin C-terminal pentapeptide)	2.3	10 [18]
Hez-PBAN ^c	1.0	2.5 [1]

^a Maximal pheromone production relative to 5 pmol Hez-PBAN injected.

^b Hex, hexanoyl; Hyp, hydroxyproline; Oic, octahydroindole-2-carboxyl.

^c Hez-PBAN, LSDDMPATPADQEMYRQDPEQIDSRKY-Phe-Ser-Pro-Arg-Leu-NH₂.

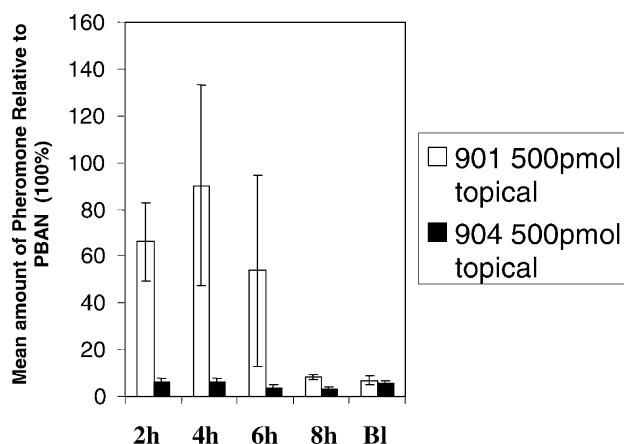


Fig. 3. Mean amount of pheromone, relative to injected PBAN (100%), produced 2, 4, 6, and 8 h following topical application of 500 pmol of Hyp-analog 901 and Oic-analog 904 to the lateral abdominal surface of adult female *H. virescens* moths.

3.4. Isolated cuticle penetration assay

We suspected that a possible reason for the inability of the analog 904 to stimulate pheromone production when applied topically at 1000 pmol and that of 500 pmol of 901 was that the amounts of the analogs that penetrated the cuticle were too small. To address this we conducted cuticle penetration studies. The results of these studies showed that less 901 than 904 penetrated the cuticle at 1, 2, and 4 h after application but the amounts recovered at 6, 8, 21, and 24 h after application were the same for both analogs (Table 2). Therefore, differential rates of penetration of the two analogs cannot explain the differences in pheromone activity. Rather, the differences in activities in the topical assays appear to stem from the differences in the potency of the two analogs (see Tables 1 and 2).

3.5. Isolated fore- and midgut penetration assays

To explore an alternate route for delivery of the analogs we studied penetration of the analogs through the fore- and

Table 2
Amounts of analogs recovered from isolated cuticle over time^a

Time after application (h)	Total amount 901 recovered (pmol; \pm S.E.)	Total amount 904 recovered (pmol; \pm S.E.)
1	3.0 (0.9) ^A	20 (2.0) ^B
2	6.0 (1.9) ^A	24.5 (2.2) ^B
4	14.4 (1.1) ^A	25.2 (1.8) ^B
6	32.6 (4.0) ^A	29.6 (1.6) ^A
8	34 (1.3) ^A	31 (1.1) ^A
21	36 (7.0) ^A	39.7 (5.0) ^A
24	38 (3.0) ^A	40.0 (3.0) ^A

Means in each row having the same superscript letter are not different in a *t*-test at $P = 0.05$ ($N = 6$ /time for each analog).

^a Numbers represent the total amounts for each time period.

midgut, reasoning that their enhanced peptidase-resistant and amphiphilic character might increase survival times in the digestive tract and penetration rates across the gut lumen wall, respectively. Unfortunately, the guts of adult moths are not of sufficient size to allow for practical delivery of peptide analog solutions into the lumen of dissected gut tissue and are too fragile. Therefore, we chose the fore- and midgut of adult cockroaches as tissues for study. The pyrokinins are known to have physiological effects on the gut of cockroaches. Significantly more of the analog 901 penetrated the midgut than did the analog 904 and the majority of 901 entered during the first 4 h (Fig. 4). In the isolated midgut preparation, 765 pmoles was recovered from the surrounding bath saline during 4-h incubation following the introduction of a total of 2.5 nmoles of the most potent of the analogs, 901, into the lumen of the midgut. Further incubation from 4 to 24 h yielded an additional 65 pmoles for a total 24 h recovery of 830 pmoles (33%; Fig. 4). A smaller total of about 300 pmoles (12%) of 904 was recovered over the 24-h incubation period. No statistically significant amounts of PBAN were recovered from midgut preparations over the 24-h incubation period, indicating that either the great majority had been degraded by peptidases in the midgut and/or was unable to penetrate through the lumen wall.

While less 904 than 901 penetrated the midgut, a greater amount of the more hydrophobic 904 penetrated the foregut preparation over 24 h (Fig. 5). PBAN did not penetrate the foregut. As can be visualized in Fig. 5, 115 and 55 pmoles, respectively, of 901 were recovered at the 0–4 h and 4–24 h

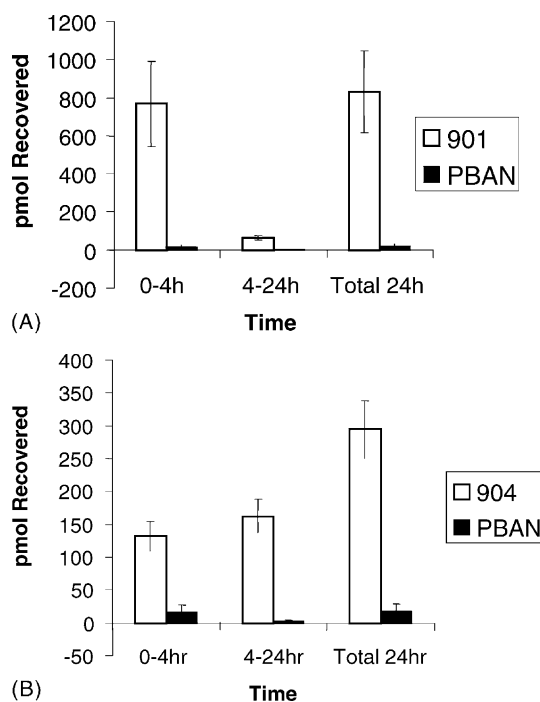


Fig. 4. Amount of Hyp-analog 901, Oic-analog 904, and the natural peptide PBAN penetrating isolated, ligated cockroach midgut preparations over indicated time periods.

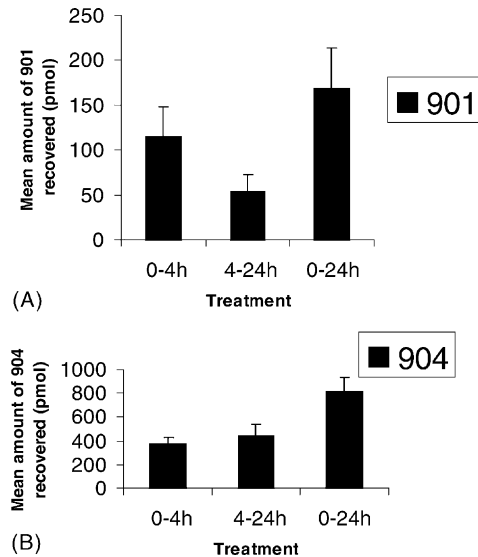


Fig. 5. Amount of Hyp-analog 901 and Oic-analog 904 penetrating isolated, ligated cockroach foregut preparations over indicated time periods.

time periods for a rather small 24 h total of 170 pmoles (7%) of 1 nmole introduced into the lumen of the midgut preparation. However, 370 and 440 pmoles, respectively, of 904 were recovered at the 0–4 h and 4–24 h time periods, respectively, for a 24 h total of 810 pmoles (32%; Fig. 5).

3.6. Pheromonotropic assay (oral)

Initial studies were conducted on the oral activity of both PBAN and the C-terminal pyrokinin pentapeptide, FTPRLamide. Starved moths were fed 50 pmol/ μ l sugar solutions of PBAN or the C-terminal pentapeptide for 15 min. Glands from females fed PBAN or FTPRLamide contained only 4.81 ± 0.6 ng ($n = 5$) and 7.18 ± 0.81 ng

($n = 5$), respectively after 2 h. These results indicated that neither PBAN nor the pentapeptide, FTPRLamide, induced pheromone production.

Following ingestion of a sugar solution containing 50 pmol/ μ l of either 901 or 904 for a period of 15 min, the pheromone production in starved female moths was monitored at 1.5, 3, 4, and 6 h post-feeding (Fig. 6). While control moths fed the native neuropeptide PBAN showed no significant pheromone production, those fed either 901 or 904 did show significant production at certain specific time intervals. The Hyp-containing analog 901 induced the release of a statistically significant 30% of the maximal titer produced by injected PBAN (PBAN maximum = 90–100 ng) at 1.5 h, but not at 3, 4, or 6 h post-feeding. Statistically significant elevated pheromone titers were also observed for 1.5, 3, and 4 h post-feeding of the Oic-containing analog 904 (Fig. 6). The analog 904 induced a statistically significant 58% of the maximal pheromone production for injected PBAN at 3 h post-feeding. The pheromone release spike for 901 occurred at 1.5 h, whereas that of 904 occurred at 3 h, indicative of a more pronounced time-release effect for the more hydrophobic analog 904 (Fig. 6).

4. Discussion

The study began with an investigation of the primary site within the C-terminal pentapeptide active core region of the pyrokinin/PBAN class of neuropeptides susceptible to tissue-bound peptidases. Incubation of the natural pyrokinin/PBAN peptide LPK (pQTSFTPRLamide) with either Malpighian tubule or hindgut tissue of the corn earworm moth *H. zea* led to rapid degradation. All of the LPK was hydrolyzed within a 30-min period. MALDI-TOF mass spectral analysis of the hydrolysis products separated by reverse-phase HPLC indicated that the primary hydrolysis

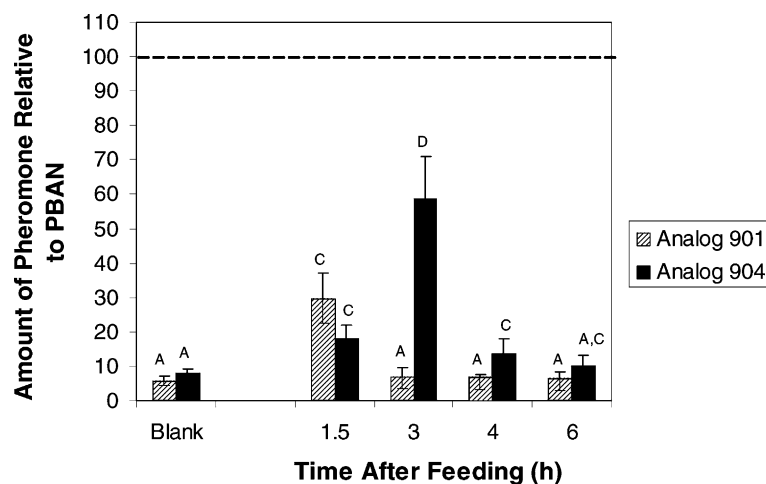


Fig. 6. Amount of pheromone, relative to injected PBAN, produced by Hyp-analog 901 and Oic-analog 904, 1.5, 3, 4, and 6 h following oral administration. Dotted line at 100% denotes maximal production of injected PBAN (positive control).

site could be identified as the peptide bond between the active core residues Pro and Arg, resulting in the cleavage of the C-terminal dipeptide. The resulting fragments are inactive in insect hindgut contractile, pheromotropotropic and pupariation acceleration assays [9]. In order to fortify the susceptible peptide bond within the core region, the adjacent Pro position was replaced with several modified residues that could provide a steric and/or physicochemical barrier to approach of the tissue-bound peptidases. The Pro residue is an integral part of the beta turn over residues TPRL which has been shown to be important for receptor interaction in a number of pyrokinin/PBAN related bioassays [8,10], and the replacements under consideration had to maintain the integrity of this beta turn. For this reason, only modified Pro residues were incorporated. In order to impart amphiphilic character to the analogs, important in enhancing cuticle and/or gut wall penetrability characteristics, the hydrophobic hexanoic acid component was attached to the N-terminus of the pyrokinin C-terminal pentapeptide core region. The analogs synthesized were Hex-Phe-Thr-[Hyp]-Arg-Leu-NH₂ (Hyp, hydroxyproline) and Hex-Phe-Thr-[Oic]-Arg-Leu-NH₂ (Oic, octahydroindole-2-carboxyl)(Fig. 1). When these analogs were incubated with either *H. zea* Malpighian tubule or hindgut tissue, they proved to be completely resistant to hydrolysis even up to 2 h, at which time the experiments were terminated (Fig. 2). As can be seen in Table 1, the intrinsic biological activity determined via injection of the analogs into adult *H. virescens* females is in the following order Hyp > Oic. Each is less active than Hez-PBAN, the native peptide. Furthermore, the Hyp analog is about 4 times less potent than the parent pyrokinin C-terminal pentapeptide, and Oic is about 50 times less potent.

Topical application studies indicated that the Hyp analog 901 could effectively penetrate the cuticle and induce production of significant amounts of pheromone in the 2–6 h time period post-application (500 pmol), reaching 90% of the maximal level of the pheromone production of injected PBAN at 4 h (Fig. 3). At 8 h, pheromone titers returned to control levels. However, no significant pheromone production could be observed at any time period between 2–8 h following topical application of even 1000 pmol of the Oic analog 904. Nevertheless, experiments to monitor the direct passage of the two analogs through isolated cuticle by HPLC demonstrated that even less of analog 901 penetrated than 904 in the 1–4 h post-application period, and equal amounts of each penetrated in the 6–8 and 21–24 h time periods. The failure of 904 to induce significant levels of pheromone following topical application appears, therefore, to be a result of low potency (Table 1) rather than an inability to penetrate the cuticle.

To investigate the possibility that these peptidase-resistant, amphiphilic pyrokinin/PBAN analogs could both survive hostile conditions within the gut and penetrate the gut lumen wall, we undertook experiments to monitor direct penetration through isolated insect fore- and midgut. These

digestive organs were dissected from the American cockroach, as the guts of adult moths are too small for practical delivery of test solutions of peptide analogs and too fragile to manipulate. The pyrokinins have been shown to have physiological effects on the cockroach gut, specifically the stimulation of spontaneous contractions [9]. In the midgut preparation, a total of 830 pmol of 901 was recovered over 24 h, representing 33% of the 2.5 nmol applied to the lumen. The great majority of 901 penetrated in the 0–4 h range. The penetration rate for 904 was much lower, with only 300 pmol (12%) recovered in a 24-h period, with about 60% of that penetrating in the early 0–4 h range (Fig. 4). No statistically significant amount of PBAN was observed penetrating the midgut preparation.

Conversely, the relative levels of performance of the two analogs were reversed in the foregut preparation. The isolated foregut preparations introduced an additional variable, as the lumen wall contains a cuticular component similar to that surrounding the exoskeleton [4]. In this preparation, amphiphilic analogs capable of penetrating the outer cuticle would be expected to show an enhanced ability to penetrate the lumen of the foregut, and demonstrate a more pronounced time-release effect. For analog 901, only a relatively small 170 nmoles (7%) was recovered over 24 h. However, a significant amount of the total (55 pmol), was recovered in the 4–24 h range (Fig. 5). A 24 h total of 810 pmol (32%) of 904 was recovered, with over half (440 pmol) penetrating in the 4–24 h range. No significant amount of PBAN could be recovered from the foregut preparations. The large percentages of the total recovered for both 901 and 904 in the 4–24 h period in comparison with the 0–4 h period provided evidence for a time-release effect (Fig. 5). This effect is more pronounced in the case of the Oic analog 904, the more hydrophobic of the two analogs.

With the successful demonstration that significant quantities of the two analogs could survive residual peptidases in the two gut preparations, and penetrate the lumen wall, the next logical step was to explore the ability of the analogs to induce in vivo pheromone production in adult female *H. virescens* moths following oral ingestion. A previous published attempt to feed Hez-PBAN to adult females of the related moth *H. zea*, reported very low and inconsistent levels of pheromone production that were not progressively dose-dependent [15]. In our experiments, no statistically significant pheromone production was observed in starved adult *H. virescens* moths 1–2 h following ingestion of a sugar solution of 50 pmol/μl of either PBAN or 1–3 h with the pyrokinin C-terminal pentapeptide core FTPRLamide. Pheromone production was monitored following ingestion of 30 μl of a 50 pmol/μl solution of either 901 or 904 at 1.5, 3, 4 and 6 h post-feeding (Fig. 6). A statistically significant increase in pheromone titer was observed only at 1.5 h post-feeding with 901 where a titer of 17% of that of injected PBAN was observed. Oral administration of the analog 904 induced statistically significant levels of pheromone at 1.5, 3, and 4 h post-feeding, but not at 6 h. Optimal pheromone

production was achieved at 3 h, with a highly significant titer of almost 60% of that of injected PBAN. The shift in the pheromone spike from 1.5 h for 901 to 3 h post-feeding for 904 is consistent with the greater time-release effect observed for the direct penetration of the more hydrophobic 904 in both the ligated fore- and hindgut preparations. Although 904 is less potent than 901 and proves to be a poor topical analog, it is clearly superior when administered orally. Analog 901 was effective at penetrating the ligated midgut but ineffective at penetrating the foregut preparation. Conversely, 904 was ineffective at penetrating the ligated midgut, but very effective at penetrating the foregut preparation. The greater effectiveness of 904 as an orally active analog may therefore be directly related to its ability to effectively penetrate the foregut, bypassing the hostile, peptidase-rich environment of the midgut. The results also suggest that the foregut can serve as a reservoir for the time-release delivery of neuropeptide analogs in pest insects.

In conclusion, fortification of the peptide bond between active core residues Pro and Arg by incorporating modified Pro analogs can enhance resistance to tissue-bound peptidases of insects. The amphiphilic Hyp analog 901 has been shown to operate as a topically active tissue-bound peptidase-resistant analog of the pyrokinin/PBAN class of insect neuropeptides in adult *H. virescens* moths. The Oic analog 904 is ineffective topically, but proves to be a superior tissue-bound, peptidase-resistant pyrokinin/PBAN analog for oral administration; clearly outperforming the orally inactive native hormone PBAN in adult *H. virescens* moths. The success of the analog 904 via oral administration may be a result of its ability to effectively penetrate (in time-release fashion) the foregut with its cuticular component, thereby bypassing the hostile environment of the midgut. The design of peptidase-resistant, amphiphilic analogs capable of effective foregut penetration can provide an effective route to the development of orally active agonist/antagonist analogs of insect neuropeptides capable of disrupting critical neuropeptide-regulated physiological processes in pest insects.

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